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Ultrasonic-assisted extraction, chemical characterization of polysaccharides from Yunzhi mushroom and its effect on osteoblast cells

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ABSTRACT

The objective of the current study was to determine the optimum ultrasonic-assisted extraction conditions for polysaccharides from Yunzhi mushroom. The optimum conditions were evaluated with fractional factorial design and optimized using response surface methodology. The ultrasonic power and the ratio of material to solvent were the most significant parameters on polysaccharides extraction. The extraction conditions were further investigated with Box–Behnken design. The fitted second-order model revealed that the optimal conditions consisted of ultrasonic power 30 W, 1:40 the ratio of raw material to solvent and extraction time 8 h. Under the optimized condition, the maximum productivity of Yunzhi polysaccharides predicted is 5.95%. The extraction productivity and purity of Yunzhi polysaccharides under the optimized extraction conditions were great higher than that of the non-optimized condition. In this study, Yunzhi polysaccharides could enhance alkaline phosphatase (ALP) activity in osteoblast cell. The present study still demonstrates that Yunzhi polysaccharides affects osteoblast cell growth in a dose-dependent manner by stimulating ALP activity.

1. Introduction

Historically, plant-derived extracts have been considered as important remedies for maintaining health, enhancing overall immune status, and prevention and treatment of chronic diseases. Recent studies have demonstrated that mushroom extracts possess a plethora of biological activities including antibacterial, antiviral, antifungal, antitumour and immuno-potentiating activities (Lau et al., 2004; Li et al., 2009; Ng, 1998; Ooi & Liu, 1999; Wasser & Weis, 1999; He, Hui, Tezuka, Kadota, & Li, 2010). Coriolus versicolor (CV), known as Yunzhi in China, is a mushroom belonging to species of the Basidiomycetes class of fungi. Its medicinal value was recorded in the Compendium of Chinese Materia Medica and Shen Non Compendium Medica thousands of years ago in China. Nowadays, its therapeutic potential has been gaining acceptance among patients worldwide (Kidd, 2000). Yun Zhi polysaccharopeptide (PSP) and polysaccharide Kureha (PSK, Krestin) are a new type of Biological Response Modifier (BRM) extracted from the deep-layer cultivated mycelia of the Cov-1 and CM-101 strains of Yun Zhi (Coriolus versicolor), respectively. Their active ingredient is a protein-bound polysaccharide with beta-1,3-glycosidic bond. They have been used in the BRM therapy of tumors and enhancement of the immune system (Wasser & Weis, 1999).

In the present study, to achieve success in extraction yield improvements, the sonic power, extraction time and ratio of solvent to raw material were evaluated for Yunzhi polysaccharides production and statistically optimized to enhance productivity. Each variable was tested based on our prior experience over a range and fixed in Box–Behnken design of RSM. The role of each variable, their interactions and statistical analysis to obtain predicted yields of Yunzhi polysaccharides was explained by applying second-order polynomial model. The data was analyzed statistically and response surface contour plots were constructed which indicated the possibility of enhancement in the production of Yunzhi polysaccharides. The analysis was done using SAS software. Then, pharmacological effect of Yunzhi polysaccharides on proliferation of osteoblast cells in bone marrow cultures was evaluated.

2. Material and method

2.1. Material

The Yunzhi mushroom, were collected form a market in Chongqing city, thoroughly washed with distilled water, freeze-dried and ground.

2.2. Extraction procedure

Ground yunzhi mushroom was refluxed with 95% ethanol at 70 °C in a water bath for 3 h to deactivate the endogenous enzymes

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and remove some soluble materials, including free sugars, amino acids and some phenols. The ethanolic mixture extract was centrifuged (3000*g*, 10 min). The ethanol extraction was washed twice with 95% ethanol. The combined extract was vacuum-dried at 60 °C for 12 h, and it was suspended in the water and sonicated at the temperature of 40–80 °C and actual sonic power of 7.2–40.3 W for 10–30 min. After rapid cooling to room temperature using ice water, the supernatant was concentrated in a rotary evaporator under reduced pressure, and then mixed with four volumes of cold 95% ethanol (12 h, 4 °C) for isolation of the polysaccharides. All experiments were performed at least in duplicate.

2.3. Experimental design and statistical analysis

Response surface methodology (RSM) was used to estimate the effect of independent variables (Power, X1; extraction time, X2; water to raw material ratio, X3) on the extraction yield (%). A Box–Behnken statistical design was employed for designing the experimental data.

The RSM was applied to the experimental data using a commercial statistical package, Design-Expert version 6.01 (Statease Inc., Minneapolis, USA). Experiments were randomized in order to minimize the effects of unexplained variability in the observed responses due to extraneous factors. The experimental design included star points, and three centre points to calculate the repeatability of the method [6]. The response functions (Y) were extraction yield. The significant terms in the model were found by analysis of variance (ANOVA) for each response. The adequacy of model was checked accounting for R^2 and adjusted-R^2.

2.4. GC–MS analysis

GC analyses were performed on an Agilent Technologies 6890 N Network gas chromatograph coupled to an Agilent Technologies 5973 Network quadrupole mass selective spectrometer and provided with a split/splitless injection port. Helium was the carrier gas, at a linear velocity of 38 cm/s. Compounds were separated on a HP-5MS capillary column (Hewlett-Packard, Avondale, PA, USA) and successively on a SPB-1 capillary column (Supelco Ltd., Bellefonte, PA, USA), both 30 m × 0.25 mm ID, 0.25 μm film thickness. Column temperature was held at 40 °C for 5 min and increased to 75 °C at 4 °C/min, then at 8 °C/min to 250 °C holding 10 min. The injector temperature was 250 °C, and samples (1 μl) were injected in the splitless mode.

The temperatures of the ion source and the transfer line were 175 and 280 °C, respectively. Positive ion electron impact mass spectra were recorded at 70 eV ionisation energy, 2 scan/s.

2.5. NMR spectroscopy

^{13}C NMR spectra were recorded with a BRUKER AM-200 spectrometer. Data points (6000–7000) were accumulated overnight at 37 °C with complete proton decoupling. The spectrum width was 5000 Hz. The gum sample (150 mg) was dissolved in deuterium oxide (1 ml). Methanol-d was added as a reference. Two-dimensional spectroscopy was applied using Correlated Spectroscopy (COSY), Heteronuclear Multiple Quantum Coherence (HMQC) and Heteronuclear Multiple Bond Correlation (HMBC) with a BRUKER AM-400 spectrometer.

2.6. Cell proliferation assay

Bone marrow cells were obtained from the femurs of 8-week-old female Kunming mice, bred in the Animal Center of the third military medicinal university. Animals were killed by cervical dislocation. All procedures were performed according to the literature (Spicer, Alonso, & Chamberlain, 2001). Cells were cultured in RPM1 supplemented with L-glutamine (4 mmol/l), penicillin (100 μg/ml), gentamycin (100 μg/ml), insulin (1 μmol/l), transferrin (5 μg/ml), selenium (0.1 μmol/l), Yunzhi polysaccharides (0.2, 0.4, 0.6 mmol/l) and containing 10% fetal calf serum. For proliferation assays the cells were were plated at a density of 2–3 × 10^5 in 96-well trays and serum deprived in media containing 1 mg/ml bovine serum albumin for 3 days. Cells were sub-confluent at the time of the assay.

2.7. Alkaline phosphatase activity

Bone marrow cells of sacrificed mice were used for organ culture as described previously (Kalimanovska, Whitaker, & Moss, 1987), in order to examine the effects of insulin on Alkaline phosphatase (ALP) activity. Briefly, cells were placed in each chamber on a wet Millipore filter. Two chambers were then placed in 7.0 ml of medium DM-170 (Kyokuto Pharmaceutical Industrial, Tokyo), with or without Yunzhi polysaccharides (0.2, 0.4, 0.6 mmol/l), in a roller tube (120 × 35 mm) and cultured for 24 h at 37 °C in an atmosphere of CO_2–air (1:19). After cultivation, the cells were homogenized and assayed for AP activity. Four experiments were carried out and the results were expressed as the mean ± standard deviation.

3. Result and discussion

3.1. Model building and statistical significance test

A 15-run Box–Behnken design with three factors and three levels, including three replicates at the centre point, was used for fitting a second-order response surface. The three centre point runs were added to provide as a measure of extraction yield. The considerable variation in the polysaccharide yield from the Yunzhi mushroom under different conditions was shown in Table 1.

The lack of fit is an indication of the failure for a model representing the experimental data at which points were not included in the regression or variations in the models cannot be accounted for random error (Montgomery, 2001). If there is a significant lack of fit which could be indicated by a low probability value, the response predictor is discarded. The lack of fit did not result in a significant P-value for selected variables, meaning that these models were sufficiently accurate for predicting the relevant responses.

Coefficient of determination, R^2, is the proportion of variation in the response attributed to the model rather than to random error

Table 1
Analysis of variances in the regression model for optimisation of polysaccharide extraction from Yunzhi mushroom.

RUN	X1 power (w)	X2 extraction time (min)	X3 ratio of solvent to raw material	*Y* (extraction rate %)
1	−1 (25)	−1 (30)	0 (8)	1.65
2	−1 (25)	1 (50)	0 (8)	2.68
3	1 (35)	−1 (30)	0 (8)	3.67
4	1 (35)	1 (50)	0 (8)	4.95
5	0 (30)	−1 (30)	−1 (6)	3.27
6	0 (30)	−1 (30)	1 (10)	3.99
7	0 (30)	1 (50)	−1 (6)	4.02
8	0 (30)	1 (50)	1 (10)	4.11
9	−1 (25)	0 (40)	−1 (6)	2.54
10	1 (35)	0 (40)	−1 (6)	4.21
11	−1 (25)	0 (40)	1 (10)	2.14
12	1 (35)	0 (40)	1 (10)	4.76
13	0 (30)	0 (40)	0 (8)	5.87
14	0 (30)	0 (40)	0 (8)	5.90
15	0 (30)	0 (40)	0 (8)	5.95

Table 2
Fit statistics for *Y*.

	Master model	Predictive model
Mean	3.980667	3.980667
R-square	98.78%	98.78%
Adj. *R*-square	96.58%	96.58%
RMSE	0.25176	0.25176
CV	6.324581	6.324581

and was suggested that for a good fitted model, R^2 should not be less than 80%. When R^2 approaches to the unity, signifies the suitability of fitting empirical model to the actual data. The lower value of R^2 shows the inappropriateness of the model to explain the relation between variables (Kuo & Lai, 2009; Yin & Dang, 2008).

Our results showed that the R^2 values for these response variables were higher than 0.80, indicating the regression models were suitable to explain the behavior. The R^2 values for extraction yield was found to be 0.9878 (Table 2). It should be noted that adding a variable to the model will always increase R^2, regardless of whether the additional variable is statistically significant or not. Thus, a large value of R^2 does not always imply the adequacy of the model. For this reason, it is more appropriate to use an adj-R^2 of over 90% to evaluate the model adequacy. The adj-R^2 values were found to be higher than 0.93 for all the responses. Higher adj-R^2 indicated that non significant terms have not been included in the model (Table 2). Moreover, coefficient of variation (CV) describes the extent to which the data were dispersed. As a general rule, the coefficient of variation (CV) should not be greater than 10%. Li et al. reported that a high CV indicates that variation in the mean value is high and does not satisfactorily develop an adequate response model. Our results showed that the coefficients of variation were less than 10% for all the responses (Table 2), representing a better precision and reliability of the conducted experiments.

The predicted second-order polynomial model was:

$$Y = 5.906667 + 1.0725 * X1 + 0.3975 * X2 + 0.12 * X3 - 1.552083 * X1 * X1 + 0.0625 * X1 * X2 + 0.2375 * X1 * X3 - 1.117083 * X2 * X2 - 0.1575 * X2 * X3 - 0.942083 * X3 * X3$$

3.2. GC–MS and NMR analysis

The Yunzhi polysaccharides showed a single and symmetrically sharp peak. According to the retention time, its molecular weight was estimated to be 2.4×104 Da. Total carbohydrate content was determined to be 95%. The polysaccharides was composed of d-glucose and d-mannose as detected by GC in the ratio of 4.4:1 (Fig. 1).

In the anomeric region of the ^{1}H NMR spectrum of Yunzhi polysaccharides, three signals occurred at 4.05, 5.10 and 4.75 ppm were observed (Fig. 2a). And accordingly in the anomeric region of the ^{13}C NMR spectrum, three carbon resonances appeared at 120.7, 96.7 and 78.0 ppm. All the results confirmed the presence of three sugar residues and their configurations. All the NMR chemical shifts were compared with the literature values (Lai, Yu, Yuen, & Chang, 2006; Rutherford, Jones, Davies, & Elliott, 1994).

The data on ^{13}C NMR analysis of the polysaccharides were showed in Fig. 2b. It had a negative response to the Bradford test and no absorption at 80 or 60 ppm in the UV spectrum, indicating the absence of protein and nucleic acid. The bands in the region of 46.12 ppm are due to the hydroxyl stretching vibration of the polysaccharides. The bands in the region of 50.42 ppm are due to C–H stretching vibration, and the bands in the region of 75.46 ppm are due to associated water. Moreover, the characteristic absorptions at 86.36 ppm and 88.26 ppm in the IR spectra indicated α- and β-configurations simultaneously existing in Yunzhi polysaccharides.

3.3. Effects of Yunzhi polysaccharides on proliferation of osteoblast cells in bone marrow cultures

The effect of Yunzhi polysaccharides on bone marrow cultures was studied by growing the cells in the presence of the hormone. The effect of Yunzhi polysaccharides on osteoblast cells proliferation was assayed. We studied the effect of Yunzhi polysaccharides on osteoblast cells proliferation at the 5th days of culture. The number of cells grown in the presence of Yunzhi polysaccharides was significantly higher on day 5 when compared with control cultures grown in the absence of Yunzhi polysaccharides. The maximal stimulatory effect on osteoblast cell proliferation was

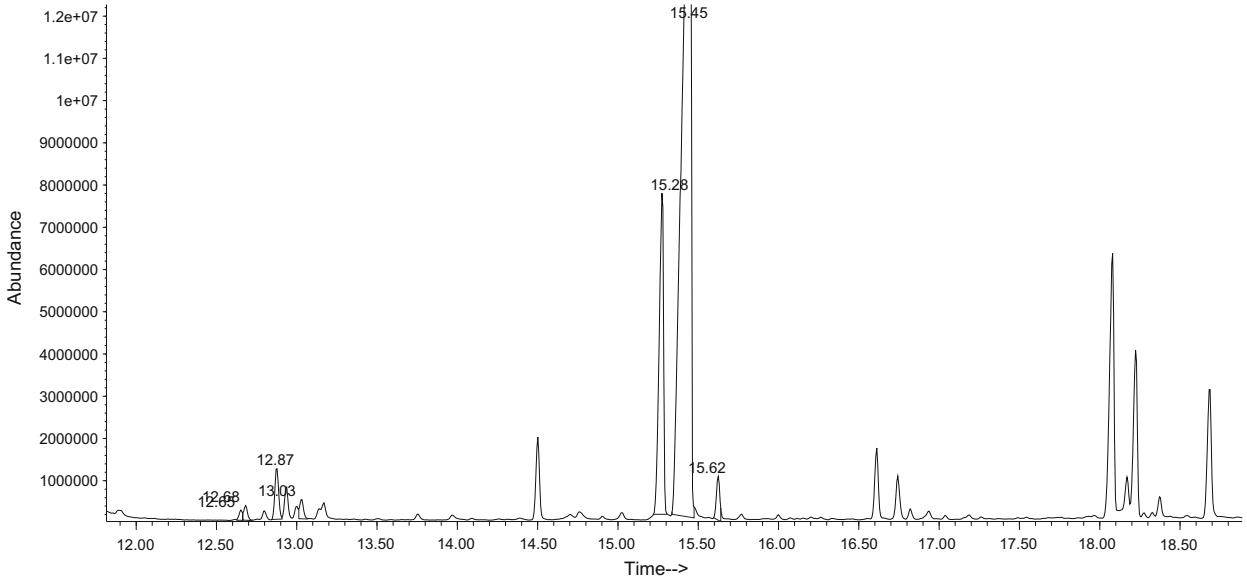

Fig. 1. GC–MS analysis of Yunzhi polysaccharides.

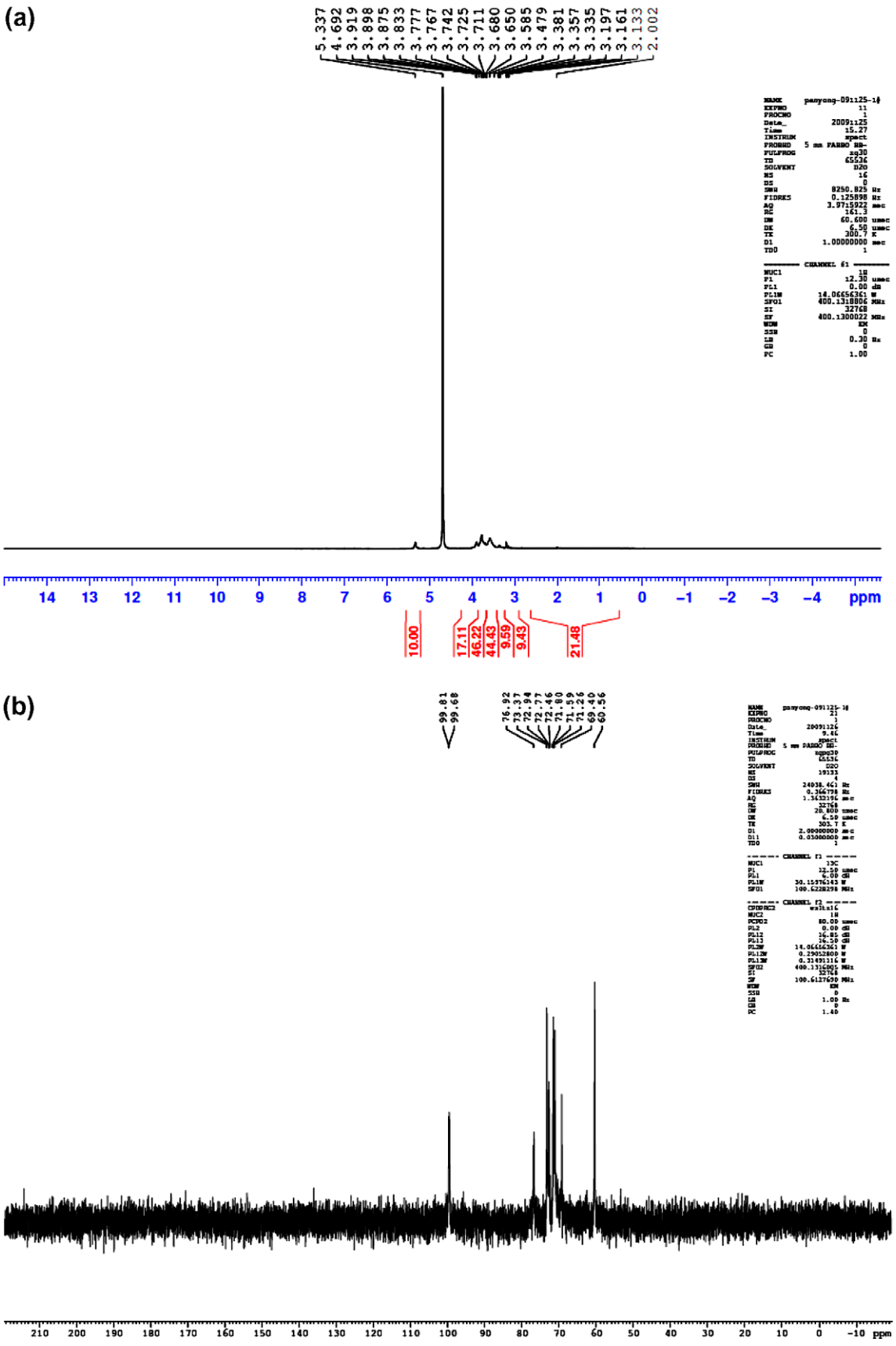

Fig. 2. (a) ^{1}H NMR analysis of Yunzhi polysaccharides; (b) ^{13}C NMR analysis of Yunzhi polysaccharides.

achieved at a concentration of 0.6 mmol/L (Fig. 3). Based on these results we propose that Yunzhi polysaccharides affects osteoblast cell growth in a dose-dependent manner by sensitizing cells towards extrinsic signals.

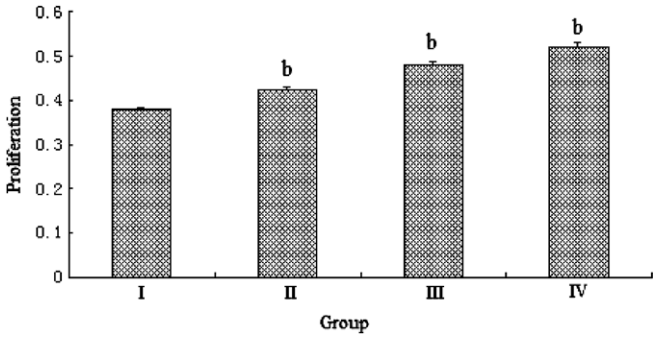

Fig. 3. Effects of Yunzhi polysaccharides on proliferation of osteoblast cells in bone marrow cultures. $^{b}P < 0.01$, compared with group I.

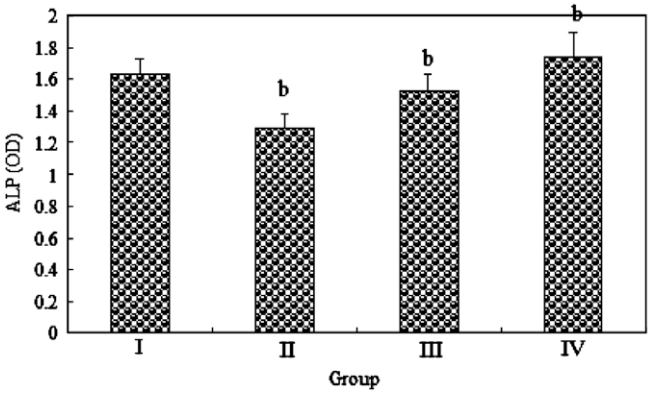

Fig. 4. Effects of Yunzhi polysaccharides on ALP activity in osteoblast cells. $^{b}P < 0.01$, compared with group I.

3.4. Effects of Yunzhi polysaccharides on ALP activity in osteoblast cells

We studied the effect of Yunzhi polysaccharides ALP activity in osteoblast cells proliferation at the 5th days of culture. In experiment, ALP activity increased ($P < 0.05$) cell proliferation above controls at each dose of Yunzhi polysaccharides (Fig. 4). The maximal stimulatory effect on ALP activity in osteoblast cell was achieved at a concentration of 0.6 mmol/L (Fig. 4). Based on these results we propose that Yunzhi polysaccharides affects osteoblast cell growth in a dose-dependent manner by stimulating ALP activity.

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